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[Contribution from the Department of Pharmacology, Medical College of Virginia]

Demethylation of Cotinine *in Vivo*[1]

By Herbert McKennis, Jr.,[2] Lennox B. Turnbull, Edward R. Bowman[3] and Einosuke Wada[4]

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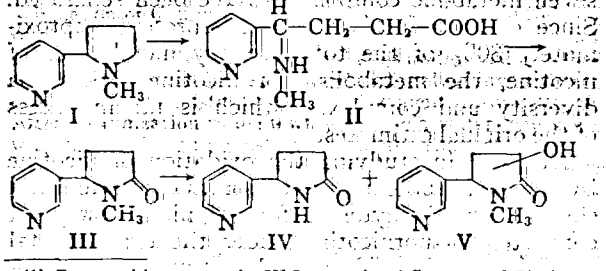
Administration of (−)-cotinine, an intermediate in the metabolism of (−)-nicotine, leads to the excretion of (−)-desmethylcotinine, hydroxycotinine and other Koenig positive material in the urine of the dog. Reductive amination of γ-(3-pyridyl)-γ-oxobutyric acid in the presence of Raney nickel yielded racemic desmethylcotinine which was resolved to give (−)-desmethylcotinine corresponding to the metabolic product.

Following the administration of (−)-nicotine the cat,[5] the dog[6–7] and the human[8,9] excrete in the urine a variety of Koenig positive materials which are not found in control urine. One of the several Koenig positive zones from paper chromatography has been found to contain cotinine.[6–9] The latter has been identified[10] chemically with the isolation of cotinine picrate,[6–9] and another has been found to contain γ-(3-pyridyl)-γ-methylaminobutyric acid,[6,7] identified after thermal cyclization into the lactam cotinine.[6,7] This cyclization to cotinine takes place spontaneously at physiological *p*H. Cotinine, through its limited toxicity, is a convenient intermediate, therefore, for exploration of nicotine metabolism. The isolation of two new nicotine metabolites now permits representation of the cotinine pathway of nicotine metabolism in the abbreviated form



For the current studies attention was focused solely on the chloroform-soluble metabolites of nicotine (I) and cotinine in dog urine. (−)-Cotinine (III) was administered intravenously to male mongrel dogs under anesthesia. The urine was adjusted to *p*H 8–9 by addition of ammonia water and extracted continuously with chloroform. Samples from evaporation of the chloroform yielded Koenig positive zones at R_f 0.61 and R_f 0.74 upon paper chromatography with ammonia–ethanol–butanol.[11] The latter corresponded in R_f value to known cotinine upon cochromatography and the former corresponded in R_f value to material obtained from both dogs[6,7] and humans[8,9] following administration of (−)-nicotine (I).

The aqueous solution of the residue from evaporation of the chloroform was placed on Dowex 50 (H^+). The Koenig positive material was eluted with *N* ammonia water and further purified by passage through Dowex 1 (OH^-) which served to remove some Koenig negative solids as well as a component with R_f 0.75. The latter has an R_f value virtually indistinguishable from cotinine in many solvent systems but differs from it by an apparent amphoteric behavior displayed on ion-exchange resins. Investigations on this component are in progress.

The effluent from the Dowex 1 (OH^-) resin containing Koenig positive bases was reprocessed on Dowex 50 (H^+) for further purification. The solids from the latter were dissolved in chloroform and chromatographed on alumina and eluted with ether containing methanol in concentration increasing from 10–75% (v./v.). The 10% methanolic eluates contained material with R_f 0.74 corresponding to cotinine. The fractions removed with 11–75% methanol, although composed of both oil and crystals showed only a positive Koenig zone at R_f 0.61. Following treatment with acetic anhydride–pyridine the foregoing mixture was chromatographed on alumina and eluted with methanolic

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(2) The authors are grateful to The American Tobacco Company and The Tobacco Industry Research Committee for generous support.

(3) Public Health Research Fellow of the National Heart Institute.

(4) American Tobacco Company Research Fellow.

(5) F. B. Owen, Jr., and P. S. Larson, *Arch. int. pharmacodyn.*, **115**, 402 (1958).

(6) H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, This Journal, **79**, 6342 (1957).

(7) H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, *ibid.*, **80**, 6597 (1958).

(8) E. R. Bowman, L. B. Turnbull and H. McKennis, Jr., *Virg. J. Sci.*, **9**, 438 (1958).

(9) E. R. Bowman, L. B. Turnbull and H. McKennis, Jr., *J. Pharmacol. Exp. Therap.*, in press.

(10) Paper chromatographic and ultraviolet absorption data have led to the suggestion (F. E. Guthrie, R. L. Ringer and T. G. Bowery, *J. Econ. Entom.*, **50**, 822 (1957), that cotinine arises from the metabolism of nicotine in insects.

(11) H. McKennis, Jr., L. B. Turnbull, H. N. Wingfield, Jr., and L. J. Dewey, This Journal, **80**, 1634 (1958).

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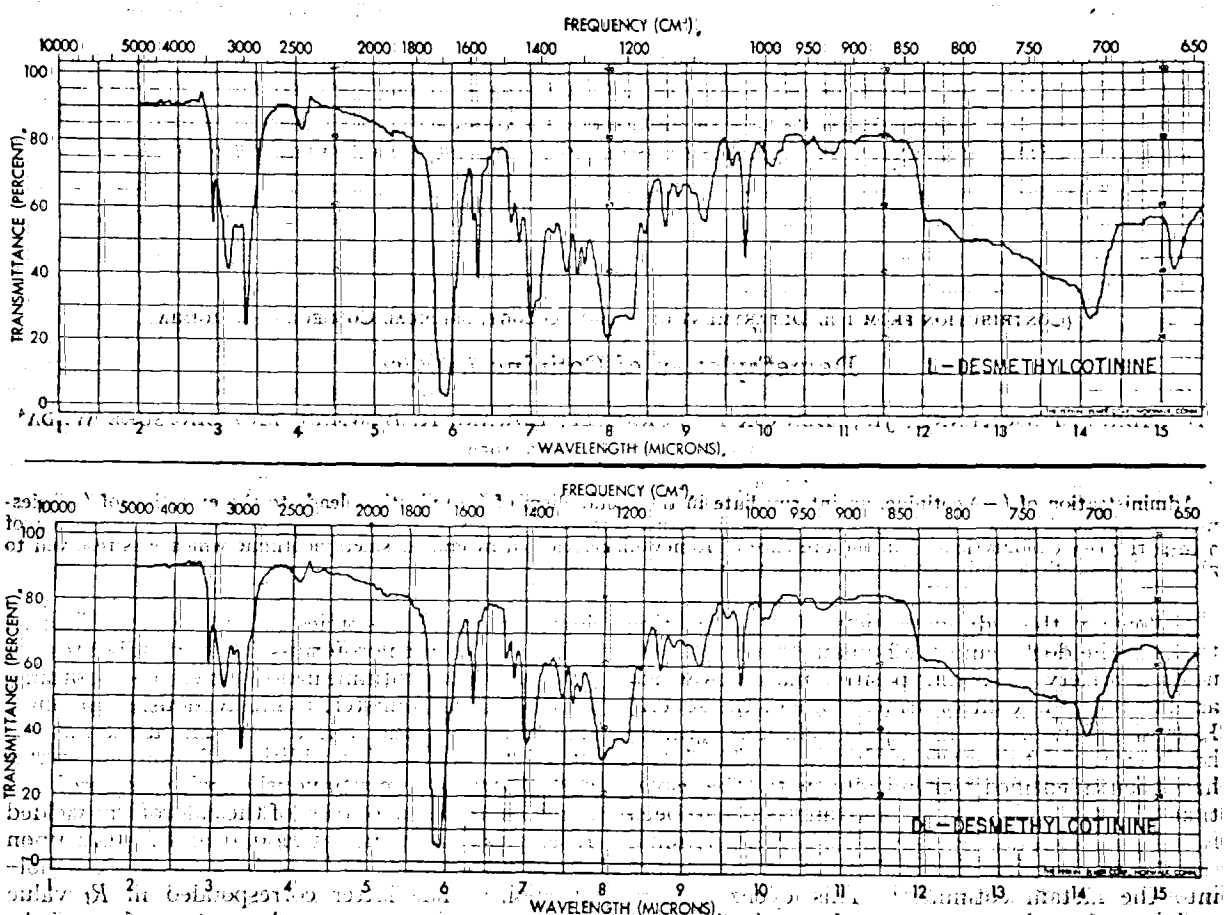

Fig. 1.—Infrared absorption spectra of natural and synthetic desmethylcotinine in chloroform solution.

ether to give two Koenig positive components (R_f 0.61 and R_f 0.75).

The material with R_f 0.61 readily crystallized from benzene to yield colorless crystals melting at 135–136°. The elementary analysis corresponded to that of desmethylcotinine (IV). The R_f value and infrared absorption spectra[12] in chloroform (Fig. 1) were in good agreement with synthetic *dl*-desmethylcotinine.

The acetyl derivative (R_f 0.75), which was obtained as an oil, yielded a yellow picrate, m.p. 166.5–170°. The elementary analysis agreed with the calculated value for the picrate of acetylated hydroxycotinine, $C_{18}H_{17}N_8O_{16}$. This has led us to adopt the trivial name hydroxycotinine for the metabolite (V). The strong Koenig positive reaction of the compound is substantial evidence to support assignment of the hydroxyl group to the pyrrolidone ring of cotinine. Exact assignment of position and structure of the molecule must await unequivocal synthetic and degradative studies.

The isolation of (−)-desmethylcotinine and hydroxycotinine as a result of the metabolism of cotinine experimentally affirms the importance of a role attributed to cotinine as an intermediate in nicotine metabolism.[11] It is also apparent that many new metabolic pathways for the degradation of cotinine must be considered. Owen and Larson[5] in their investigation of chromatograms of the urine of dogs after administration of C¹⁴(−)-nicotine suggested the presence of three major and possibly four minor metabolites of nicotine. As a result of the present and previous studies,[6,7] seven metabolic components have been separated. Since on a molar basis these represent approximately 30% of the total urinary metabolites of nicotine, the metabolism of nicotine presents a diversity and complexity which is far in excess of the original estimates.

Hucker[13] in studying the oxidation of nicotine by fortified rabbit liver microsomes concluded that since no formaldehyde was formed, nicotine was not converted to nornicotine under the experimental conditions. If oxidative demethylation is involved in the metabolism of nicotine to desmethylcotinine, our isolation of desmethylcotinine points to the presence of a pathway for demethylation which may involve other cellular components of the liver[14,15] or other organs.[16] In the event, however, that demethylation occurs by transmethylation, rather than oxidative demethylation, formaldehyde cannot be anticipated as a primary metabolic product or serve as an absolute indicator of de-

(12) Kindly determined by Mr. J. Scott Osborne of The American Tobacco Company, Research Laboratory.

(13) H. B. Hucker, *J. Pharmacol. Exp. Therap.*, **122**, 33a (1958).

(14) T. Akira, *Folia Pharmacol. Japon.*, **53**, 553 (1957).

(15) E. Werle and R. Müller, *Biochem. Z.*, **308**, 355 (1941).

(16) See for example: A. W. Miller, Jr., and P. S. Larson, *J. Pharmacol. Exp. Therap.*, **109**, 218 (1953); E. Werle and E. Uschold, *Biochem. Z.*, **318**, 531 (1948).

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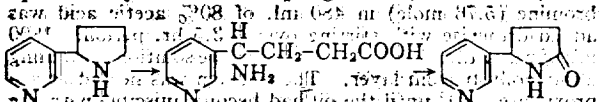
methylation.[17] Irrespective of the type of demethylation involved, the simplest explanation for the formation of desmethylcotinine involves only participation of cotinine or γ-(3-pyridyl)-γ-methylaminobutyric acid (II).

Werle, *et al.*,[18] as a result of studies on the metabolism of nicotine considered the possibility that nicotine was demethylated to give nornicotine (VI). Chemical verification of this proposal would suggest additional metabolic pathways to desmethylcotinine with nornicotine and possibly γ-(3-pyridyl)-γ-aminobutyric acid (VII) as intermediates and no involvement of cotinine. Experiments to test such a possibility are now in progress.



To facilitate further metabolic work improved procedures have been developed for the synthesis of cotinine and desmethylcotinine. The latter has been resolved successfully with *d*-10-camphorsulfonic acid. This resolution provides both optical antipodes. Reduction of the racemic mixture provided[11] synthetic routes to nornicotine. Racemic cotinine has been reduced to racemic nicotine.[19] (−)-Cotinine upon reduction with lithium aluminum hydride in the present study yielded (−)-nicotine. This reduction supplies experimental confirmation of the previous conclusion[7] that in the metabolism of nicotine to (+)-γ-3-pyridyl-γ-methylaminobutyric acid and (−)-cotinine the absolute optical configuration of (−)-nicotine is retained.

Acknowledgments.—The authors are grateful to James H. Smith, Covie E. See and Jeffrey S. McKennis for assistance.

Experimental[20]

Isolation of Cotinine Metabolites.—(−)-Cotinine (5.9 g.) was administered intravenously[21] (100 mg./kg.) during an 8-hour period to two male mongrel dogs under Dial anesthesia. Urine was collected through an indwelling catheter during the administration and subsequent 10-hr. period. After alkalinization to *p*H 9 with ammonia water, the urine was extracted continuously with chloroform. The residue from evaporation of the chloroform (3.1 g. of brown gum) yielded two Koenig positive zones (R_f 0.74 and R_f 0.61, ammonia–ethanol–butanol, R_f 0.48 and R_f 0.30, formic acid–*sec*-butyl alcohol–water). Upon cochromatography these zones corresponded to those previously obtained from dogs treated intravenously with nicotine or orally or intraperitoneally with cotinine. The brown gum was dissolved in 10 ml. of water and adjusted to *p*H 1.5. The solution was placed on Dowex 50 (H^+). After a water wash, Koenig positive material, which was retained on the resin, was eluted with *N* ammonia water. The residue (1.5 g.) from evaporation of this solution was dissolved in dilute ammonia and placed on a Dowex 1 (OH^-). The residue from concentration of the effluent was again placed on Dowex 50 (H^+). Elution with 0.5 *N* ammonia water served to remove 738 mg. of material which again gave Koenig positive zones (R_f 0.74 and R_f 0.61, ammonia–ethanol–butanol). A chloroform solution of the mixture was placed on acid-washed alumina and eluted with ether containing methanol increasing from 5–75% (v./v.). The 5–10% methanolic eluates contained material with R_f 0.74 corresponding to cotinine (299 mg.). The fraction removed with 11–75% methanol, although composed of both oil and crystals (359 mg.), showed only one Koenig positive zone (R_f 0.61, ammonia–ethanol–butanol; R_f 0.30, formic acid–*sec*-butyl alcohol–water).

Isolation of (−)-Desmethylcotinine and Acetylated Hydroxycotinine.—The mixture of oil and crystals was dissolved in pyridine containing an excess of acetic anhydride and allowed to stand overnight. The residue after removal of reagents at the vacuum pump showed the original R_f value plus a zone at R_f 0.75 (ammonia–ethanol–butanol). Rechromatography on acid-washed alumina served to separate the two fractions. The R_f 0.75 material appeared as a colorless oil from concentration of the eluates in 10% methanolic ether (75 mg.). The oil was treated with an excess of picric acid in methanol to yield a crystalline picrate which was purified for analysis by recrystallization from 95% ethanol and drying at 70° and 1 mm. (m.p. 166.5–170°).

Anal. Calcd. for $C_{18}H_{17}N_7O_{10}$: C, 46.67; H, 3.70; N, 15.12. Found: C, 46.82; H, 3.98; N, 15.29.

The analysis corresponds to that expected from the picric acid salt of acetylated hydroxycotinine.

Concentration of the eluates in 11–50% methanolic ether yielded 150 mg. of colorless crystals. For analysis these were recrystallized from benzene, m.p. 135–136°, and dried at 70° under diminished pressure, $[\alpha]^{25}_{5461}$ −60.7° (*c* 1.65, methanol).

Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.22; N, 17.27. Found: C, 66.34; H, 6.31; N, 17.3.

Synthetic (−)-desmethylcotinine did not depress the melting point of the compound and showed the same R_f values upon chromatography on paper.

(−)-Nicotine from (−)-Cotinine.—A solution of cotinine (4.5 g.) in 100 ml. of dry tetrahydrofuran was added dropwise to 90 ml. of tetrahydrofuran containing 1.2 g. of lithium aluminum hydride at 0°. After refluxing for 40 hr., the solution was treated with 70 ml. of acetone[22] and then with 100 ml. of 5 *N* NaOH. The aqueous phase was separated and washed twice with chloroform. The combined organic phases were dried over anhydrous sodium sulfate and concentrated to 5.5 g. of light-colored oil. The oil was dissolved in chloroform and placed on a column of acid-washed alumina (200 g.). By elution with ether containing successively increasing amounts of methanol, two Koenig positive fractions were obtained. The minor fraction, obtained last from the column, consisted of 0.31 g. of oil, R_f 0.89 (ammonia–ethanol–butanol). Nicotine, the major fraction, (2.3 g.) (R_f 0.84), was converted in methanol to the dipicrate, m.p. 223–224.5° (4.9 g.). The mixed melting point with authentic material was not depressed. The purified dipicrate (2.37 g.) was treated with 0.5 *N* HCl (50 ml.). After removal of picric acid by filtration, the aqueous phase was made alkaline with ammonia and extracted with chloroform. The chloroform extracts were concentrated to an oil. The latter was dissolved in methanol and treated with decolorizing carbon. The filtrate was concentrated in a stream of nitrogen to yield 0.38 g. of nicotine (dried under diminished pressure), $[\alpha]^{25}_{5461}$ −162.5° (*c* 2.75, acetone).

The *dl*-Desmethylcotinine.—γ-(3-Pyridyl)-γ-oxobutyric acid[11,23] (15 g.) was hydrogenated at 80 atmospheres and approximately 130° in 300 ml. of absolute ethanol containing 30 g. of ammonia and approximately 5 g. of Raney nickel for 7 hr. The filtrate from the catalyst was concentrated to an oil. The latter was dissolved in dilute ammonia water. An extraction with three portions of benzene (150 ml. each) served to remove some red colored material and facilitated a final extraction with five portions of chloroform (150 ml. each). After drying over anhydrous sodium sulfate the chloroform extract was concentrated to 10.2 g. of oil which

(17) E. B. Keller, J. R. Rachele and V. du Vigneaud, *J. Biol. Chem.*, **177**, 733 (1949).

(18) E. Werle, H. Schievelbein and D. Spieth, *Arzneimittel-Forsch.*, **6**, 322 (1956).

(19) S. Sugawawa, T. Tatsuno and T. Kamiya, *Pharm. Bull. (Japan)*, **2** [1], 39 (1954).

(20) Microanalyses by Weiler and Strauss and Spang Microanalytical Laboratory.

(21) Q. McKennis, E. R. Bowman and H. McKennis, Jr., *Toxicol. Appl. Pharmacol.*, **1**, 61 (1959).

(22) H. McKennis, Jr., and G. W. Gaffney, *J. Biol. Chem.*, **175**, 217 (1948).

(23) R. N. Castle and A. Burger, *J. Amer. Pharm. Assn. Sci. Ed.*, **43**, 163 (1954). These authors reported that they were unable to convert the keto acid into desmethylcotinine by catalytic reduction in the presence of ammonia.

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solidified on standing. The latter was recrystallized twice from benzene to give 7.2 g. of desmethylcotinine monohydrate, m.p. 65–68°. Admixture with an authentic sample[11] produced no melting point depression. The product at this stage is sufficiently pure for resolution. Further purification may be effected by chromatography of a benzene solution on alumina and then elution with 5–90% methanolic ether. The fractions from methanolic ether (20–90%) yield a crystalline form from benzene, m.p. 89–91°, which differs in melting point from the previously described[11] anhydrous or monohydrated compound. Upon standing in the open air the compound in a few hours forms a monohydrate which melts at 66–67°.

(−)-Desmethylcotinine.—*dl*-Desmethylcotinine (1.80 g.) and 2.50 g. of *d*-10-camphorsulfonic acid were dissolved in 200 ml. of warm ethanol. The cooled solution was treated with 100 ml. of ether and placed in an ice-bath. Crude (−)desmethylcotinine *d*-10-camphorsulfonate precipitated and was separated from mother liquor A. Recrystallization from 100 ml. of ethanol yielded 1.33 g. of the purified salt, m.p. 222–225°, and mother liquor A-1. A solution of the salt in 5 ml. of water was made alkaline with ammonia and then extracted with five portions of chloroform (7 ml. each). The combined chloroform extracts were dried over anhydrous sodium sulfate and then evaporated to yield colorless crystals (0.35 g.). These melted at 135–137.5° after one recrystallization from benzene (0.31 g.), $[\alpha]^{25}D$ −60.9° (*c* 1.12, methanol), $[\alpha]^{25.5}_{5461}$ −60.5° (*c* 2.81, methanol).

(+)-Desmethylcotinine.—The mother liquor A from the crude *d*-camphorsulfonic acid salt of (−)-desmethylcotinine (corresponding to 10.8 g. of *dl*-desmethylcotinine) was concentrated to dryness to give 16.6 g. of crude *d*-camphorsulfonic acid salt of (+)-desmethylcotinine. To a solution of the salt in 200 ml. of ethanol was added 100 ml. of ether. Upon cooling in the ice-bath 9.1 g. of salt was deposited. The latter was decomposed with ammonia water and 3.03 g. of crude (+)-desmethylcotinine (m.p. 68–110°) was deposited. The latter (2.51 g.) was dissolved in 140 ml. of absolute ethanol and treated with 3.58 g. of *d*-camphorsulfonic acid. Upon cooling to 0° the solution yielded 5.0 g. of salt and mother liquor B. The salt was combined with another lot obtained from evaporation of mother liquor A-1 (from recrystallization of *d*-10-camphorsulfonic acid salt of (−)-desmethylcotinine above). The combined lot was dissolved in 300 ml. of ethanol The solution deposited crystals upon cooling in the ice-bath. This crop then was recrystallized from 400 ml. of ethanol and finally from 200 ml. of ethanol to give 1.5 g. of optically pure *d*-10-camphorsulfonic acid salt of (−)-desmethylcotinine, m.p. 227–228°, and combined mother liquors C. The salt gave 0.3 g. of (−)-desmethylcotinine, m.p. 134.5–136°.

Mother liquor B was treated with 300 ml. of ether. Upon standing overnight in the refrigerator the solution yielded 0.45 g. of salt, m.p. 196–198°. The latter upon treatment with ammonia and chloroform gave (+)-desmethylcotinine, 80 mg. after recrystallization from benzene, m.p. 133–135°, $[\alpha]^{25}D$ +60.3° (*c* 1.16, methanol).

Mother liquor C upon evaporation to dryness gave 13.0 g. of salt. This was dissolved in 400 ml. of ethanol and then cooled in the ice-bath. Crystals, 5.37 g., were collected. The filtrate was treated with 400 ml. of ether and cooled to 0°. Salt (5.25 g., m.p. 193–210°) was collected and decomposed to give 2.06 g. of crude (+)-desmethylcotinine. Recrystallization from benzene afforded 1.0 g. of (+)-desmethylcotinine, m.p. 133–137°.

γ-(3-Pyridyl)-γ-*N*-methylbenzamidobutyric Acid.—A solution of 15 g. of γ-(3-pyridyl)-γ-methylaminobutyric acid monohydrate and 2.9 g. of sodium hydroxide in 70 ml. of water was treated (always keeping the *p*H above 9) with a total of 410 ml. of 10% sodium hydroxide and 72 g. of benzoyl chloride in alternate portions with cooling and shaking. The mixture was diluted to one l. and acidified with 2 *N* HCl (200 ml.). After removal of benzoic acid by ether extraction, the solution yielded the benzoyl derivative by direct precipitation at *p*H 6. The yield was 10.85 g. (50.9%), m.p. 173–175°. The mother liquor contains cotinine, unreacted methylamino acid and a small additional amount of the benzoyl derivative. For analysis a sample was recrystallized from ethanol, m.p. 175.5–176.5°, $[\alpha]^{26}_{5461}$ −127.8° (*c* 5, methanol).

Anal. Calcd. for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.57; H, 5.96; N, 9.17.

Lamberts and Byerrum[28] prepared the benzoyl derivative by a similar procedure in 25% yield, m.p. 170°.

Dibromocotinine Hydrobromide Perbromide.—To a solution of 88 ml. (0.538 mole) of (−)-nicotine (Black Leaf "99") in 400 ml. of 80% acetic acid, 198 ml. of technical bromine (5.76 mole) in 480 ml. of 80% acetic acid was added dropwise with stirring over a 3.5 hr. period. 1800 ml. of water then was added to the orange solution containing a dark oily bottom layer. The solution was heated to approximately 80° until the oil had become miscible with the solvent. Upon cooling to room temperature the solution deposited orange needles. The solid was removed by filtration and washed with water. On drying in air the material, m.p. 140–145°, weighed 163 g. (70%, based on the empirical formula $C_{10}H_9N_2OBr_2 \cdot HBr \cdot Br_2$). On several successive runs the yields ranged between 60 and 75%. In contrast a yield of 39% was obtained by following the method of Pinner.[24]

(−)-Cotinine.—To 2500 ml. of 50% acetic acid 200 g. (0.34 mole) of dibromocotinine hydrobromide perbromide was added. After the addition of 125 ml. of concentrated HCl, 250 g. of zinc dust was added portionwise with stirring over a 3-hr. period. The solution was stirred for an additional hour and then, after removal of unreacted zinc, adjusted to *p*H 8–9 with concentrated ammonia water.[25] On cooling the alkaline solution was extracted three times with one l. portions of chloroform. Upon evaporation of the chloroform the residue spontaneously crystallized to give 56.4 g. of cotinine which had a boiling point of 145–150° under 0.7 mm. pressure. Lamberts and Byerrum reported a boiling point of 170–175° under approximately 1 mm. pressure. Cotinine obtained above or prepared by the method of Pinner was found usually to be chromatographically pure as disclosed by paper chromatography on Whatman No. 1 paper in the ammonia–butanol–ethanol and formic acid–*sec*-butyl alcohol–water systems.[26] However, certain samples of cotinine were found to contain an impurity at R_f 0.61 (cotinine R_f 0.75) as disclosed[27] in the butanol (100 ml.)–water (20 ml.)–ethyl *p*-aminobenzoate (0.3 g.) system[29] and also at R_f 0.61 in the ammonia–butanol–ethanol systems. Distillation of these samples at the temperature and pressure reported above failed to remove the impurity. However, this can be separated from the cotinine by alumina chromatography using methanol–ether as the eluting solvent.

RICHMOND, VIRGINIA

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(25) Suggested by Mr. H. N. Wingfield, Jr., The American Tobacco Company, Research Laboratory.

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(28) B. L. Lamberts and R. U. Byerrum, *J. Biol. Chem.*, **233**, 939 (1958).

(29) Dr. R. A. Clayton of The American Tobacco Company, Research Laboratory, kindly called our attention to the procedure using butanol–water–ethyl *p*-aminobenzoate.

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